Mouse Models to Study the Interaction of Risk Factors for Human Liver Cancer

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Abstract

Each of the risk factors for human liver cancer ( aflatoxin exposure, hepatitis B virus-associated liver injury, p53 loss, p53/erbB249 mutation, and male sex) also increases the incidence of hepatocellular carcinoma (HCC) in mouse models of hepatocarcinogenesis. Neonatal mice, partially hepatectomized adult mice, and p53-deficient mice each have a higher hepatocyte proliferation rate, are less able to detoxify AFB1, and form more DNA adducts than do normal wild-type controls. However, transgenic hepatitis B surface antigen mice, expressing hepatitis B surface antigen under control of the albumin promoter ( alb/psx), are able to detoxify AFB1 at the same level as do wild-type mice. Thus, AFB1-induced HCC development in neonatal mice and p53−/− mice may be due to “immature” carcinogen metabolism, whereas increased HCC in transgenic hepatitis B virus mice may be due to promotion effects of increased proliferation. Future studies will explore the effects of modifying factors on the development of HCC.

Introduction

Risk Factors for HCC. HCC accounts for only 1% of new cancers detected per year and 2% of cancer deaths in the United States. However, HCC is the predominant cause of cancer mortality in Sub-Saharan Africa and Southern China (2–4). It causes 65–75% of all cancer deaths in males and 30%–55% of cancer deaths in females in Mozambique and in some provinces of Southern China (4–8). Epidemiological studies have identified infection with HBV or HCV and contamination of peanuts or grain with AFB1 as two major, possibly synergetic risk factors in these areas (3, 7, 8). AFB1 is a major public health problem in developing countries, where long-term food storage at high heat and humidity encourages growth of the mold.

Of particular interest in regard to the relative importance of chronic hepatitis and AFB1 is the geographical distribution of HCC in China. This distribution is very uneven and does not follow closely the incidence of chronic hepatitis (9–11). The areas of high frequency of HCC are the coastal regions in Jiangsu province and southwards in the Guanxi Autonomous Region (Fig. 1). This high risk is dependent on the relationship of the source of drinking water to the exposure to aflatoxins produced on moldy grain by growth of Aspergillus species. In high-risk areas, the incidence of HCC is highest in individuals who drink water that washes over grain that is being cured by exposure to sunlight on the roofs of houses. (Ref. 9; Fig. 2). The extraordinarily high risk of HCC in residents of Penghu Island of Taiwan compared with other Taiwanese may be attributable to their heavy exposure to aflatoxins and a high HBV carrier rate (12, 13). All of 181 consecutive cases of pathologically diagnosed HCC in Quidong, China, had evidence of HBV infection, whereas only 5% had evidence of HCV infection (14). HBV infection appears to sensitize hepatocytes to the carcinogenic effects of aflatoxin. In contrast, in the United States HCV and HBV are essentially equal risk factors for HCC, and the effects of hepatitis are enhanced by heavy alcohol consumption and diabetes (15). The contribution of aflatoxin exposure to HCC incidence in the United States is unknown.

Mouse Models of Human Cancer. Advances in genetic engineering have lead to the development of many mouse models of cancer that simulate human cancers. A major program for such models is supported by NIH and AACR (16). Most of these models explore the interaction of different oncogenes and tumor suppressor genes in the development of different kinds of cancer. For example, coexpression of an oncogene under control of an immunoglobulin promoter, such as c-myc, an activator of cell proliferation, and bcl-2, an inhibitor of apoptosis, greatly increases the risk of acute B-cell leukemia in double transgenic mice (17), a situation similar to B-cell lymphomas in humans (18). Transgenic mouse models of hepatic growth regulation and hepatocarcinogenesis have similarly shown a role in hepatocarcinogenesis for liver-specific activation of oncogenes, such as c-myc and H-ras, and expression of growth factors, such as transforming growth factor-α and insulin-like growth factor-II (19, 20). The genetic manipulation of transgenic mice to express genes involved in the development of cancers, as well as knockout mice that do not express genes that interfere with cancer development, permits the design of mouse models to examine the role of multiple genetic and environmental factors in cancer. The application of transgenic and knockout mouse models for the study of the mechanisms of the interaction of the risk factors for human HCC is the subject of this review.

Transgenic HBsAg ( alb/psx) Mice

Aflatoxin Effects in alb/psx Transgenic HBV Mice. The effect of AFB1 on development of HCC in mice with chronic liver injury has been examined using transgenic mouse lineage 50–4 expressing the HBsAg. In these mice the HBV BgIII A fragment is linked to the albumin promoter (21), directing expression to liver cells. This fragment contains the entire HBV envelope-region open reading frame including three in-phase translation-initiated codons, which represent, respectively, the NH2 termini of the large, middle, and major envelope polypeptides of HBV, as well as the x-protein ( alb/psx). Transgenic mouse lineages with a C57BL/6 background carrying the alb/psx gene express different levels of the HBsAg polypeptide in the cytoplasm of their hepatocytes but do not express the x-protein. Transgenic mice of lineage 50–4 used in our studies do not secrete HBsAg, but rather store it in the endoplasmic reticulum, resulting in liver dysplasia at about 3–5 months of age, followed by severe prolonged hepatocellular injury and death, regenerative hyperplasia beginning at ~6 months of age, and aneuploidy, oval-cell proliferation, nodule formation, and eventually development of HCC at 15–18 months of age in males and 22–24 months in females (22). Liver cell injury, inflammation,
and regenerative hyperplasia place large numbers of hepatocytes at risk for the development of transforming mutations and eventually HCC (23). Male mice are more severely affected throughout the course of the process, as they store more surface antigen at an earlier age, have more injury and dysplasia, and uniformly develop HCC, whereas female mice store less HBsAg, have less injury and dysplasia, develop HCC later and at a lower incidence (22), and may even be able to repair the cellular defect and regenerate their livers to a normal appearance after 20–22 months (24). Ongoing studies in our laboratory show that regeneration of HBsAg negative foci, which do not express mRNA for HBsAg, begins at ~7 months of age and occurs in close association with the bile ducts in the portal zone (Fig. 3, A and B), suggesting that these foci may arise from liver progenitor cells located in the duct or periductal zone (25). Preliminary data indicate that the cells in these foci have lost the HBsAg transgene, but additional analyses must be done to confirm the nature of this loss. Later HbsAg-negative tumors appear in association with these foci in male mice (Fig. 3, C and D), supporting the concept that these cancers may arise from stem cells (26). In contrast to 100% HCC development in males, many female 50–4 mice are able to replace large portions of the damaged HBsAg-containing cells with relatively normal appearing HBsAg-negative tissue, and survive until natural death without development of HCC.

On the other hand, exposure of line 50–4 female mice to DEN or AFB1, known hepatocarcinogens in species other than mice (adult), results in more rapid development of premalignant changes (dysplasia and nodules) and HCC than in 50–4 female mice not exposed to DEN or AFB1 (27). However, exposure to phenobarbital, a known promoter, does not increase HCC incidence, indicating that HBsAg storage injury and phenobarbital do not synergize in this effect. This would be expected, as neither is considered a carcinogen. In addition, Zoran Ilic in our laboratory showed that alb/psx 50–4 lineage mice fed diets high in cadmium appear to develop HCC more rapidly, with males more prone to developing tumors than females (24). These results indicate that these alb/psx mice react to carcinogens and dietary factors in a similar manner as do humans infected with HBV.

Testing for oncogenes and tumor suppressor genes has revealed no mutations in HCCs arising in 50–4 HBsAg transgenic

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**Fig. 1.** Hyperendemic areas of HCC in China. The gray shaded areas are high-risk areas, which have a high humidity and temperature conditions favorable for growth of Aspergillus species, which produce aflatoxin, and endemic infection with HBV.

**Fig. 2.** Source of drinking water, and morbidity and mortality from HCC in high incidence areas of China. Morbidity and mortality per 100,000 people per year. The incidence of HCC is related to the degree of contamination of the water with aflatoxin produced by moldy grain curing on the roofs. Many of the HCC cases under these conditions are relatively young men (9).
mice (28). However, although no p53 mutations have been found, about one-third of HCCs occurring after AFB1 injection (postweaning) have amplification of the mdm2 gene (29). This interesting observation deserves additional study.

The p53 Gene. The p53 gene is one of the most frequent targets for genetic alterations in human cancers (30, 31). Although it has many functions, it acts primarily as a tumor suppressor (32, 33). The product of the wild-type p53 gene is a phosphoprotein that migrates at Mr 53,000 on a denaturing polyacrylamide gel. The NH2 terminus of the protein contains an acidic transactivation domain that interacts with cellular transcription cofactors to facilitate transcription from p53-responsive elements (34, 35). The COOH-terminal end of the p53 protein contains a basic domain, which is essential for nuclear localization (36) and for nonspecific DNA binding (37). The central part of the p53 molecule is most sensitive to point mutations and is the specific DNA-binding domain (38, 39).

Wild-type p53 may transactivate hundreds of cellular promoters, such as those of mouse muscle creatine kinase, p53, mdm2, wild-type p53 activated fragment 1 (p21), growth-arrested and DNA damage-inducible gene products (GADD45), as well as apoptosis-promoting factors, such as Bax, which block the action of Bcl-2 (40–43). p53 binds to and inactivates several oncoproteins such as the SV40 T antigen, the adenovirus E1b 55k protein, and the human papilloma virus E6 protein (44–46). Thus, p53 may act via its various properties to block cell division, enhance DNA repair, increase apoptosis, or block growth-activating proteins. Mdm2-p53 binding may autoregulate p53 expression and modulate the activity of p53 in the cell (42, 47, 48), especially during development, when down-regulation of p53 is essential (49, 50).

Another property of p53 that has direct relevance is the effect of the specific p53 protein has on activation of the PCNA promoter. Under some experimental circumstances wild-type p53 increases the production of PCNA by binding to its promoter (51, 52). However, wild-type p53 actually depresses the PCNA promoter in other systems (53), and the activation that is reported is dose-dependent in the systems in which p53 activates PCNA expression (51). As indicated below, decreased p53 levels in p53+/− and p53−/− null mice are associated with an increase in the number of liver cells in G1 containing PCNA, as well as an increase in mitoses. Thus, it appears that normal p53 levels in the liver are not sufficient to activate PCNA expression, but that decreasing p53 (in p53 null mice) is associated with increased PCNA, because more cells are entering the cell cycle.

Aflatoxin, p53 Mutations, and HCC. A mutation in codon 249 of the p53 gene occurs with high frequency in HCC occurring in high-incidence areas of the world (8, 54). Fifty-eight percent of HCCs from Qidong, China, and 53% from Mozambique contain an AGG to AGT mutation at codon 249 of p53, resulting in the replacement of Ser by Arg in the mutant protein (55). The frequency of this mutation correlates with aflatoxin exposure (2, 8, 55). In the United States, only 3 of 19 HCC tumor tissues tested contained AFB1-lysine conjugates, and none had the p53ser249 mutation associated with aflatoxin exposure (56). In one study, exposure of p53 DNA in vitro to AFB1 is associated with formation of adducts at the third base of codon 249 (57); however, in another study using hepatoma cells in vitro, adduct formation was more frequent in other sites (58). HCCs in woodchucks infected with HBV and exposed to aflatoxin did not show p53 mutations (59). No p53 mutations in the region corresponding to human p53 codon 249 were found in AFB1-induced preneoplastic foci in rats (60), unless proliferation was induced by PH (61). No p53 mutations are seen in carcinogen-induced mouse liver tumors (62). Regardless of whether or not p53 mutations occur in HCCs of other species, the availability of p53 knockout mice as well as the introduction of a specific p53 mutation (p53ser246), equivalent to human p53ser249, into transgenic mice, provides model systems by which to study the role of loss and mutation of this gene in hepatocarcinogenesis.

p53 Null Mice. p53 null mice have been developed by Lawrence Donehower, Baylor College of Medicine (Houston, TX; Ref. 63). Homozygous p53−/− mice develop lymphomas at the age of 5–6 months, and so are unsuitable for longer-term hepatocarcinogenesis experiments. However, heterozygous p53+/− mice survive for up to 2 years of age and provide a model for determining the effect of reduced p53 in experiments directed toward determining the importance of p53 loss in hepatocarcinogenesis (64).
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HCC in p53 Null X Transgenic alb/psx F1 Mice. To determine the effect of AFB1 on alb/psx mice with loss of one allele of p53, p53+/− male C57BL/6 mice were bred to 50–4 mice to obtain alb/psx mice with one p53 allele loss. Each animal was sacrificed at 13 months of age for determination of tumor presence using the grading system of Becker (65). The presence of more than one risk factor increased the incidence of HCC over that seen with one risk factor (Fig. 4). The highest incidence was seen in mice with each of the risk factors (male sex, HBsAg expression, and loss of one p53 allele (p53+/−) and AFB1 exposure. In each group males had significantly more tumors than did females. Fewer tumors were seen if any one of the three factors was not participating, i.e., if the mouse was female, if both p53 wild-type alleles were present, if HBsAg was not expressed, or if AFB1 was not given. For example, 100% of male mice with each of the other three factors had grade III or higher tumors. If both p53 alleles were present the incidence dropped to 62%; if HBsAg was not expressed, the incidence was only 14%; if AFB1 was not given the incidence was 25%.

In addition, in some of the alb/psx and p53+/− mice exposed to AFB1, some of the tumors were of a histological grade higher than had been seen previously (27, 65), such as cholangiocarcinomas, poorly differentiated adenocarcinomas, mixed hepatocellular carcinomas, and undifferentiated blastocarcinomas (grade V tumors). This suggests that the interaction of these risk factors can produce cancer of higher malignancy than that seen with one risk factor. No tumors above grade I were found in control HBV−, p53+/+ mice exposed to AFB1. This was expected, as it takes >13 months for tumors to appear in normal C57Bl mice exposed to AFB1 before weaning. Thus, each of the risk factors contributes to increased incidence of HCC, and each acts to increase synergistically, the effect of the other risk factors.

p53 Mutations

Properties of Mutant p53 Products. The p53 gene product is an unstable protein with a half-life of 5–20 min (66, 67). Over 75 different mutations may occur at various codons of p53 in liver cancer (8). Mabs have been generated that show selective binding to wild-type or mutant p53 proteins. In particular, mab pAb246 binds the wild-type p53 protein, whereas mAb pAb240 does not bind wild-type, but does bind most, although not all, mutant p53 proteins (68, 69). The protein products of the mutant forms of the p53 gene have markedly varied properties. Some are quite different from the wild-type, whereas others are similar to it. Mutants generally have an extended half-life of 12–20 h (70). Because of this prolonged half-life, the presence of a mutant p53 protein may be assumed after immunolabeling using cross-reacting antibodies in transgenic mice with mutant p53 protein, if wild-type p53 protein is not normally present. p53 protein mutants that recognize pAb240 are unable to bind to SV40 T antigen (SV40TAg) but do bind to heat shock proteins (71). Many of these mutations may also result in a nonfunctional protein as they fail to suppress colony formation in vitro when transfected into transformed cell lines (72) and fail to transactivate the mouse muscle creatine kinase promoter (40). Oncogenic forms of mutant p53 inhibit wild-type p53-regulated gene expression (73).

p53ser246 Mutation. To determine the effect of the specific p53ser249 mutation associated with aflatoxin induced human HCC, Nader Ghebranious, in collaboration with Gigi Lozano and Brian Knoll, constructed the equivalent mutation for the mouse under the control of the albumin promoter to produce transgenic mice expressing this mutation in the liver. Because of a three codon deletion in the mouse p53 gene, as compared to the human, the p53ser246 mutant in the mouse is equivalent to the human p53ser249 (74). To compare the properties of the product of a mutant mouse p53ser246 to the wild-type protein and for production of transgenic p53ser246 mice, he introduced a two-nucleotide change in the mouse gene at amino acid position 246 (Fig. 5) using the recombinant PCR mismatched-primer method (75). This p53 mutation resulted in the same change, and Arg to Ser substitution, as in the human p53 gene at position 249.

The protein product of this mutant mouse p53ser246 has properties similar to those of the wild-type protein, when tested by binding to monoclonal antibodies pAb246 and pAb240, SV40TAg, or heat shock protein 70 [(Hsp70); Ref. 74]. However, it has mutant-type transforming properties when tested for colony formation using an osteosarcoma cell line (SaOs-2) (74). It is not active, as is wild-type p53, in transcription activation of the muscle creatine kinase (MCK) promoter. Thses properties are the same as those found in the p53trp248 product of the p53 mutation associated with the Li-Fraumeni syndrome (76). Although less is known about the human p53ser249
product associated with HCC carcinoma, the mutant murine p53ser246 protein shares the known properties of the human gene product.

**HCC in p53ser246 Transgenic Mice.** An albumin promoter/p53ser246 construct was made using a truncated mouse albumin promoter from Dr. Richard Palmiter, University of Washington, Seattle, (77) and transgenic mice expressing the p53ser246 mutant in the liver were generated (78). Of seven lineages tested, two, including lineage 24, had strong nuclear staining for p53 in the liver for the first 3 weeks of life; this pattern in not seen in wild-type mice and is due to expression of the mutant gene. The presence of high-grade tumors in transgenic p53ser246 mice bred to p53 null mice with or without AFB1 injection is shown on the right-hand side of Figure 4. The presence of the p53ser246 mutation increased HCC incidence in p53+/− mice exposed to AFB1, and in transgenic HBV p53+/+ mice exposed to AFB1 (78). In each set of mice expressing the mutant p53, there were more high-grade HCCs than in the age-matched controls not expressing the mutant. In a direct comparison of p53, there were more high grade HCCs than in the age-matched mice exposed to AFB1 (78). In each set of mice expressing the mutant p53, there were more high grade HCCs than in the age-matched controls not expressing the mutant. In a direct comparison of p53, there were more high grade HCCs than in the age-matched mice exposed to AFB1 (78).

**Proliferation of Hepatocytes and HCC**

**p53-Deficient Mice.** p53 is a cell-cycle control protein. Because of the possibility of an influence of p53 status on proliferation and ploidy of hepatocytes that might be related to susceptibility to hepatocarcinogenesis, the ploidy and proliferation in the livers of the p53 null mice and transgenic p53ser246 mice were determined by Li Yin in our laboratory. The results are summarized in Fig. 6 (79). The deletion of the wild-type p53 gene, from p53+/+ to p53+/− to p53−/− mice, is associated with decreases in polyploidy and increases in the number of PCNA-labeled cells in each phase of the cycle, as well as with increases in the number of cells incorporating triiated thymidine. Thus, loss of p53 appears to remove “blocks” in the control of the cell cycle of hepatocytes, allowing cells to continue to proliferate in adult mice at an age when most hepatocytes are arrested in G1, and are polyploid. Whereas cell proliferation is increased in p53 null mice, no “compensatory increase” in apoptosis is found. The net result of an increase in mitoses with no increase in apoptosis is an increased cell density in the liver, as reflected in increased numbers of small liver cells in the perportal zones of the p53 null and p53ser246 mice. One of the properties of wild-type p53 is to increase transcription of dozens of proapoptotic genes. Thus, one of the reasons why there is not an associated increase in apoptosis in the face of increased proliferation in the p53 null mice may be that loss or mutation of p53 indirectly inhibits apoptosis.

**Alb/p53ser246 Transgenic Mice.** The presence of the p53ser246 mutation appears to have little effect on the increasing polyploidy with aging in p53+/− or p53−/− mice, in the number of PCNA-positive cells in the S/M phase or in the number of cells incorporating triiated thymidine, relative to these numbers in p53 null mice; however, p53ser246 significantly increases the number of PCNA-containing cells in G1 (Ref. 79; Fig. 6). Thus, p53ser246 expression in the liver appears to inhibit progression of the cell cycle from G1 to S, but not entry of cells into G2. The mechanism of this inhibition is not known. If this inhibition does occur, then the number of cells that enter the cycle must be increased in the p53−/− or p53−/− mice to the same extent in the presence or absence of the p53ser246 mutation, but the number of cells that progress through G1 at any given time must be decreased in the mutant mice (blocked in G1). The next question as to how the loss of p53 affects control of the cell cycle was addressed in collaboration with Thomas Friedrich, Paul Yang, and Ming Sun at Albany Medical College (80).

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**EFFECT OF p53 ON CELL CYCLE OF HEPATOCYTES**

![Graph showing the effect of p53 on cell cycle of hepatocytes.](cancersres.aacrjournals.org)
Control of Proliferation in the Liver of p53 Null Mice. As a first step in defining the mechanism of increased hepatocyte proliferation in p53-deficient livers, we compared wild-type and p53-deficient mice for differences in the expression of cell-cycle regulatory proteins. Given that normal adult hepatocytes are in G₀ or early G₁, we focused on the cyclins, cyclin dependent kinases (cdks) and their inhibitors that are known to act in the G₀ to G₁ and G₁ to S phase transitions (80). Determination of cyclins and cdks by Western blot analysis revealed modest increases over the amounts seen in wild-type mice in the expression of cyclins D2, D3 and A, and cdks 2 and 4; this pattern is consistent with the increased percentage of cycling hepatocytes in p53 deficient mice.

On the basis of the well-documented transactivation of the p21cip1 promoter by p53, we expected that p21 expression might be lower in p53-null mice. Although the average p21cip1 protein level in p53-null livers was slightly lower than in wild-type livers, the difference was not significant. The absence of a significant difference in the low levels of p21 in p53-deficient livers is in agreement with the report that only very small amounts of p21 mRNA exist in p53+/+ and p53−/− adult mouse livers (81). In addition, expression of the cdk inhibitor p16ink4a in p53 was not changed in p53-null mouse livers.

Unexpectedly, we found that the major differences between wild-type and p53-deficient mice are a reduction in the cdk inhibitor p27kip1 and an increase in pRb. Passage from G₁ to S phase is dependent on the phosphorylation and inactivation of the retinoblastoma protein, pRb, by cyclin D/cdk4 and cyclin E/cdk2 complexes. Analysis of p27 in the same samples demonstrated a correlation between decreased p27 expression and increased pRb expression. Although p53 acts mainly through induction of expression of cell cycle inhibitors, to the best of our knowledge no clear association of p27 with pRb has been reported. Thus, this appears to be a novel observation related to how p53 controls hepatocyte proliferation and susceptibility to chemical hepatocarcinogens in mice. The reduction of p27 in p53-deficient livers is greater than can be accounted for by decreased p27 only in the low percentage of proliferating cells. Therefore, the quantity of p27 is likely to be reduced in both nonproliferating and proliferating hepatocytes.

Studies on other cell types have suggested that the absence of p27 is responsible for a lack of responsiveness to growth-inhibitory signals. As a result, there may be continued cell proliferation and delayed differentiation (82–85). Viewed in this context, low levels of p27 in p53-deficient livers may result in proliferating hepatocytes that fail to respond to the growth-inhibitory signals that are required to maintain homeostasis. It is the persistence of proliferating hepatocytes after the normal cessation of proliferation at 3 weeks of age that may explain the increased susceptibility of p53-deficient mice to chemical hepatocarcinogenesis (64). The next question addressed is how does proliferation affect AFB1 metabolism?

**AFB1 Metabolism.** AFB1 belongs to a group of closely related diferunocumarin compounds synthesized by the common fungal molds *Aspergillus flavus* and *A. parasitius*. AFB1 requires microsomal oxidation to form the reactive AFOB that is the ultimate hepatocarcinogen (reviewed in Ref. 86). The extent of covalent binding of AFOB to cellular DNA when measured in vivo correlates with the carcinogenic effect of AFB1. AFOB may be conjugated enzymatically to GSH by GST, which is critical for the pathway of AFB1 detoxification (Ref. 87; Fig. 7). The balance of the rate of activation (exo-epoxide production) to inactivation by GST conjugation is a critical indicator of the selectivity of GST isoenzymes toward conjugation of AFOB.

**Proliferation and AFB1 Hepatocarcinogenesis.** Both DEN and AFB1 are carcinogenic in neonatal mice, but adult mice are almost completely resistant (90, 91). One of the features in common between neonatal and older p53 deficient mice is the presence of proliferating hepatocytes. Continued hepatocyte proliferation is believed to contribute to development of HCC in *alb/pxs* transgenic mice (23). The “immature” phenotype of the proliferating hepatocytes in the young adult p53 deficient mice may explain why these mice are more susceptible to chemical hepatocarcinogenesis.

**Effect of Age on Proliferation and AFB1 Metabolism.** To determine the relationship, if any, between proliferation and AFB1...
metabolism, Thomas Shupe measured proliferation and AFB1 metabolism after administration of radiolabeled AFB1, in wild-type mice of various ages and after PH, as well as in p53/H11001/H11002 and alb/psx (HBsAg) transgenic mice. Fig. 8 shows proliferation 

(KI-67 labeling), AFB1 adduct formation, GST levels, and GSH levels in wild-type mice of various ages. Neonatal wild-type mice, which are susceptible to AFB1 hepatocarcinogenesis, have up to 200-fold higher numbers of KI-67-positive cells, form ~200 times lower levels of GST, and have much higher levels of KI-67 labeling.

Fig. 8. Proliferation, AFB1-DNA adduct formation, and GST and GSH levels in p53+/+ male mice at various ages. Neonatal mice at 4 days and 10 days of age have much higher proliferation rates in the liver, form AFB1/DNA adducts at a much higher level, and have much lower levels of GST than do adult mice. GSH levels do not change significantly from 10 days of age to adulthood. ND, not determined.

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PROLIFERATION (KI-67), AFLATOXIN B1/DNA ADDUCTS, GLUTATHIONE S-TRANSFERASE LEVELS AND GLUTATHIONE LEVELS IN WILD-TYPE MICE AT VARIOUS AGES

Fig. 9. Proliferation, AFB1-DNA adduct formation, GST levels, and GSH in age-matched wild-type and transgenic HBV p53+/+ male mice. *, the low level of AFB1/DNA adducts in the p53−/− mouse livers may be due to the presence of leukemic cells, which would contribute to the amount of DNA in the test preparation that is not available for adduct formation.
the level of carcinogen-DNA adducts, and have >100-fold lower levels of GST than do adult wild-type mice.\(^2\) On the other hand, the liver GSH level and P450 activity are not significantly different (data not shown). These results indicate that neonatal mice may be more susceptible to AFB1 because of an inability of proliferating hepatocytes to detoxify the carcinogen at the same rate as adult mice.

**Effect of PH on Proliferation and AFB1 Metabolism.** This hypothesis was tested in adult mice after PH. There is a decrease in hepatic GST in 120-day-old partially hepatectomized mice from 0.27 mmol/mg protein in control mice to 0.06 mmol/mg protein and an increase in AFB1-DNA adduct formation from 5.9 pmol AFB1/mg DNA to 350 pmol AFB1/mg after PH. Sham PH, in which the abdomen is opened and the liver is massaged slightly but not cut, results in lowering of GST to almost the same level as after PH (to 0.08 mmol/mg protein), but there is a 10-fold higher rate of AFB1 adduct formation (31.4 pmol AFB1/m DNA) than occurs after PH. There is an increase in KI-67 labeled nuclei, from <1/×40 microscopic field in normal mice to 8/×40 field after sham hepatectomy, to 104/×40 field after PH. These results suggest that reduction of GST alone, as seen after sham PH, does not fully explain the increase in AFB1 adduct formation. Thus, both decreased GST activity and changes in the chromosomes associated with mitosis may determine the degree of AFB1/DNA adduct formation.

**Effect of Expression of p53 on Proliferation and AFB1 Metabolism.** A comparison of hepatocyte proliferation and AFB1 metabolism in wild-type mice (p53+/+), heterozygous p53 knockouts (p53+/-), and homozygous p53 knockout mice (p53-/-) is shown in Fig. 9. The ~10-fold increase in AFB1-DNA adduct formation in p53+/- heterozygous mice is much greater than expected, in view of the only 4-fold increase in proliferation, and <2-fold reduction in GST levels, based on comparison to the results shown above in newborn wild-type mice (Fig. 8) and after PH. In contrast, adduct formation in p53-/- mice is essentially the same as in wild-type mice, although the amount of proliferation is ~10-fold higher. This latter result may be due to leukemic infiltrate present in the homozygous p53 null mice, which would contribute to the total DNA; however, the DNA of the leukemic cells would not be available for AFB1 adduction. This possibility is now being checked.

**Effect of HBsAg Expression on Proliferation and AFB1 Metabolism.** On the other hand, there is no difference in the amount of AFB1/DNA adduct formation or in GST levels for the transgenic alb/psx (HBsAg) mice and wild-type mice at ages when liver damage and restitutive proliferation are greatest in the alb/psx mice (Fig. 9). In the experiments in which induction of HCC was increased by AFB1 exposure in the alb/psx mice, the carcinogen was given at 10 days or 30 days of age. At neither of these times was there evidence of increased proliferation or decreased GST in the transgenic alb/psx mice. Thus, the synergism between AFB1 exposure and HBsAg expression is most likely due to a promoter effect of hepatocyte injury and chronic proliferation in the transgenic alb/psx mice rather than to an effect on carcinogen metabolism. This mechanism is especially likely because AFB1 exposure occurs within the first month of age, before the HBsAg storage injury would be expected to have an effect on proliferation. An unanswered question at this time is how HBsAg storage injury by itself is able to induce HCC in the absence of exposure to known carcinogens, in view of the inability to demonstrate oncogene abnormalities in the resulting tumors. This is most likely caused by oxygen radical damage during the chronic phase of liver injury (8, 92).

**Future Studies.** The availability of the genetically altered mouse models for the study of the interaction of risk factors for human HCC cancer may be used for a number of future studies. For example, the effect of drugs such as oltipraz, which inhibits HBV replication and transcription of the HBV gene in vitro in infected cells and induces wild-type p53 (93), or chlorophyllin (94), which forms molecular complexes with carcinogens, blocking their bioavailability and reducing urinary excretion of aflatoxin-N (7) guanine, on AFB1 carcinogenesis, could be examined. It would be of interest to see whether oltipraz works in p53-deficient mice, because its activity might be p53 dependent. Another future study is to determine the effect of the HBV x-protein, a trnascriptional activator (95, 96) believed to be critical for HBV infection (97, 98). HBV x-protein also activates nuclear factor κB transcriptional activator and abrogates p53-mediated apoptosis (8).

**Summary and Conclusions.** The major risk factors for primary HCC in humans (aflatoxin exposure, hepatitis associated liver injury, p53 loss or mutation, and male sex) are shown to interact in mouse models of hepatocarcinogenesis. The genetically altered mice used in this work include lineage 50-4 transgenic alb/psx mice expressing HBsAg (produced by Frank Chisari, Scripps Research Inst., La Jolla, CA), p53 knockout mice (produced by Larry Donehower, Baylor College of Medicine, Houston, TX), and p53ser246 transgenic mice expressing the mutant p53ser246 under the control of the albumin promoter (produced by Nadar Ghebranious, our laboratory). Each of the risk factors for human HCC also increased the incidence of HCC in these mice. AFB1 or HBsAg endoplasmic reticulum storage injury in transgenic mice will produce HCC in the absence of other factors, but coexistence of these factors or association with p53 loss or mutation additionally increases the incidence of HCC. Administration of AFB1 to male transgenic HBsAg mice lacking one wild-type p53 allele and expressing the mouse p53ser246 mutation (equivalent to the human p53ser249 mutation) produces the highest HCC incidence. Lower rates are seen when any one of the risk factors is not present. The increase of HCC associated with AFB1 exposure correlates with proliferation of liver cells after HBsAg storage injury, and in p53-deficient and p53ser246 mutant mice. Neonatal mice are susceptible to AFB1 hepatocarcinogenesis until ~3 weeks of age, when proliferation of hepatocytes drops rapidly to adult levels. However, the livers of the transgenic HBV mice show marked proliferation in response to ongoing liver injury after 7 months of age, and some hepatocytes of p53+/− mice continue to proliferate beyond 3 weeks of age. Reduced levels of p27kip1 have been found in the livers of p53-deficient mice; this may contribute to increased proliferation. Measurement of AFB1 metabolism and adduct formation shows that neonatal mice and partially hepatectomized adult mice are less able to detoxify AFB1, and they form more DNA adducts than do normal adult mice. In addition, the livers of p53+/− mice are also less able to detoxify AFB1 than wild-type age-matched controls. On the other hand, the livers of transgenic HBV mice are able to detoxify AFB1 at the same level as seen in wild-type mice. It is concluded that increased HCC development in neonatal mice is due to “immature” carcinogen metabolism, whereas increased HCC in p53+/−, p53ser246, and transgenic HBV mice is due to promotion effects of increased proliferation. However, increased inability of p53-deficient or mutant mice to prevent mutations and genomic instability associated with AFB1 exposure cannot be ruled out. These mouse models may be used to study factors that modify hepatocarcinogenesis in various experimental systems. The availability of transgenic mice expressing the HBV

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\(^2\) T. Shape, and S. Sell. Low hepatic glutathione-S-transferase and increased hepatic DNA adduction contribute to tumorigenicity of AFB1 in newborn and partially hepatectomized mice, submitted for publication.
x-protein permits future studies in mouse models of the interaction of this factor with the other risk factors for human HCC.

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References
10. Weischenfeldt, J., Reuter, A., and Kjaer, K. The hepatitis B virus X protein permits future studies in mouse models of the interaction of this factor with the other risk factors for human HCC.

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MOUSE MODELS FOR RISK FACTORS OF HUMAN LIVER CANCER

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59. Rivkina, M., Cote, P. J., Robinson, B. C., Tennant, B. C., and Marion, P. L. Absence of mutations in the p53 tumor suppressor gene in woodchuck hepatocellular carci-


77. Hagemann, G. H., and Neumann, H-G. Differences in Aflatoxin B1 - susceptibility of rat and mouse are correlated with the capability in vitro to inactivate Aflatoxin B1 epoxide. Carcinogenesis (Lond.), 2: 229–308, 1981.


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